Investigation of Toe Tip Necrosis Syndrome in Feedlot Cattle using Biomechanical Testing and Imaging

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By

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Abstract

Toe Tip Necrosis Syndrome (TTNS) is a debilitating disease affecting the lower hind limb of cattle characterized by necrotic bone, wear or rounding of the hoof and breakdown of the white line region. This breakdown is thought to be the cause of TTNS, though this is unclear. The overall aim of this research was to investigate the hypothesis that mechanical damage and loading is the cause of the white line separation, which could ultimately lead to TTNS.

Objective 1 of this research aimed to identify if loading is associated with white line separation and subsequently TTNS. Eleven diseased specimens and ten healthy specimens were loaded in a materials testing system against an acrylic plate. A camera, located underneath the hoof, imaged the apical region to identify the area of white line separation. Specimens were imaged using a High-Resolution Peripheral Quantitative Computed Tomography (HR-pQCT) scanner with a contrast agent to assess the volume of separation. Objective 2 investigated whether mechanical damage under a fatigue loading regime would induce white line separation. Ten healthy pairs of specimens were tested (10 healthy, 10 with simulated damage) under fatigue loading for 36,000 cycles at 1000 N.

The results from Objective 1 indicated a positive association between loading and white line separation in diseased hooves. However, healthy hooves showed no separation. These findings support the hypothesis that mechanical loading is involved in TTNS etiology or disease exacerbation and acceleration.

Findings from Objective 2 indicate no association between cycle count and white line separation. These results suggest that white line separation (and subsequent TTNS) may not be due to wear or prolonged repetitive loading. These results indicate that another loading parameter or a biological event likely initiates white line separation and ultimately TTNS.

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Glossary

Term	Definition
Abrasion	Refers to the removal of material through contact with a surface
Apical	Refers to the tip of something, in context this refers to the tip of the toe
Distal	Pertains to different ends of an extremity; proximal end of extremity is the end situated farthest from the center of the body
Keratin	Fibrous biological material formed from proteins forming the hoof.
Lateral	Situated at or extending to the side
Load	This refers to the application of force, weight or pressure applied by something (mechanical testing apparatus)
Medial	Situated at or extending to the middle
Necrosis	Refers to death of the cells in the tissue or organ
Osteitis	Inflammation of bone(cortical)
Osteomyelitis	Inflammation of bone(trabecular) or bone marrow usually due to infection
Potting	procedure of securing and fixing a specimen with a bonding agent to test the specimen
Proximal	Pertains to different ends of an extremity; proximal end of extremity is the end situated nearest the center of the body
Ventral	Pertaining to the underside
White line	Region of the hoof where the horn material meets the sole material

Chapter 1: Introduction

1.1 Overview

The disease toe tip necrosis syndrome (TTNS) causes lameness in feedlot cattle across Western Canada. This disease is classified by separation of the white line, P3 necrosis, and toe abscesses in the hind limb of feedlot cattle (1-7). Lameness has been estimated to cost the industry $42.5 million annually, and any reduction in lameness would significantly impact this evaluation (5, 8). Currently, treatment methods include antibiotics and paring (slicing the apical region) of the toe in the early stage of the disease. Amputation of the affected region is done if a later stage of the disease is found, but most cases do not fully recover. Many cases have the disease in more than one hind limb.

The leading hypothesis as to the cause of TTNS suggests that mechanical abrasion and loading may be the cause of white line separation, which leads to this disease (1-3, 5). Therefore, an understanding of the effect of loading and mechanical abrasion on the lower hind limb is essential for understanding TTNS. At present, there is limited information available regarding lower hind limb loading effects, with results limited to a few materials testing studies and finite element analyses (9-12).

To our knowledge, the literature contains no information regarding the amount of separation of the white line in diseased hooves, with or without loading. As well, there is no information available regarding the effects of abrasion on white line separation (e.g. an agitated animal dragging their hoof across the ground). This is important as loading and damage may increase susceptibility for separation (1-7).

The goal of this thesis research was to investigate the effect of loading and mechanical abrasion on bovine hooves to improve our understanding of the TTNS. Methodology and tools were developed to show the effects of loading on bovine hooves. These tools were then used to quantify white line separation in diseased hooves, thereby increasing our understanding of the disease as well as disease etiology.

1.2 Functional Anatomy

Bone and Joints

There are five bones (three major) and three joints that comprise the bovine lower hind limb. The lower portion of the limb is connected through the metatarsophalangeal joint also known as the fetlock joint (5, 13, 14). This joint is comprised of the distal end of the metatarsal connecting to the proximal phalanx bone or the P1 bone of two of the four digits which make up the lower limb. The fetlock joint is important for lower limb motion and weight bearing. The fetlock joint can be seen in Figure 1-1. The proximal sesamoid bone, sits just ventral to the dew claws (5, 13, 14). This bone supports the major tendons in the lower limb. The sesamoid bone is not weight bearing. The dew claws are non-weight bearing digits in the lower hind limb; hanging off the plantar side of the lower hind limb (5, 13, 14). The second major bone in the lower hind limb is the middle phalangeal bone or the P2 bone. There are two of these bones in the lower hind limb one for either hoof. These act as weight bearing bones which connect the hoof to the limb (5, 13, 14). The P2 bone provides the hoof with its mobility. There is another small sesamoid bone located inside the hoof capsule called the distal sesamoid bone or navicular bone. This bone also provides support to the tendons and the hoof while bearing weight. The third major bone in the lower hind limb is the distal phalangeal bone or the P3 bone shown in Figure 1-1. This is a weight bearing bone and is connected via the interphalangeal joint to the P2 bone. P3 is entirely cortical bone. There are many soft tissues made up of collagen fibres that connect the P3 bone to the hoof capsule. This tissue is responsible for transferring the load from the hoof capsule to the bone (5, 13-15). These bones can be seen in the Figure 1-1 below.

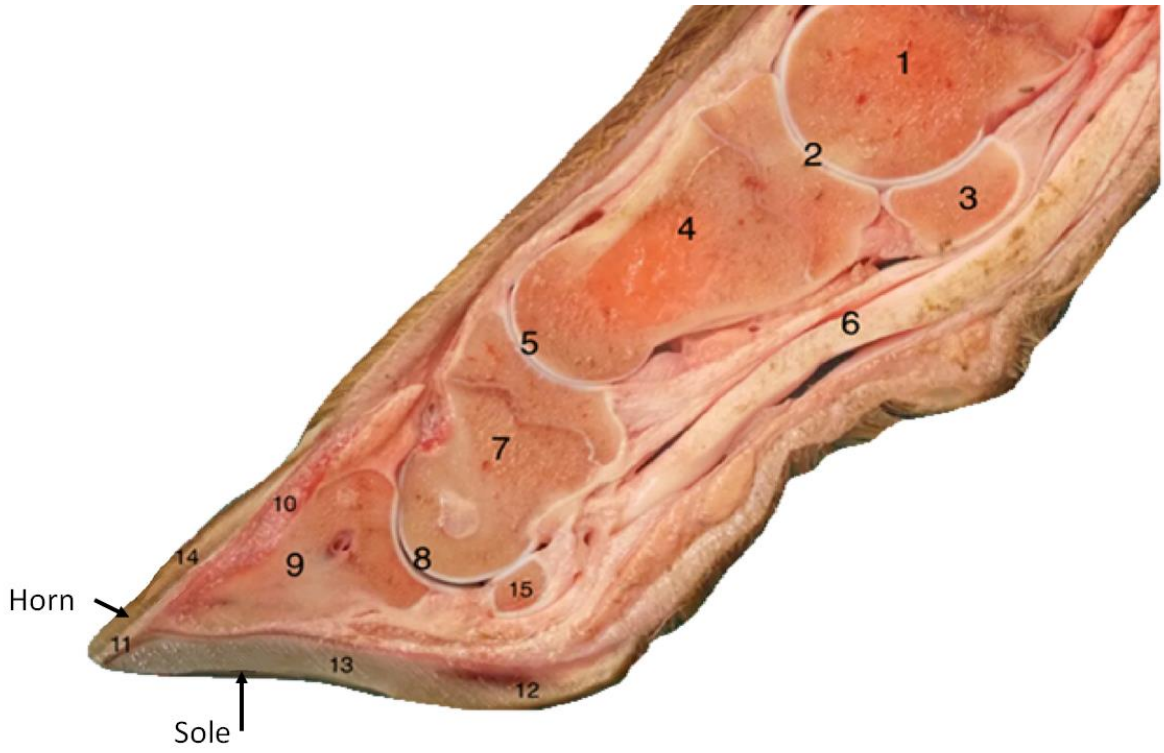

Figure 1-1 Sectioned bovine hoof showing anatomy of the lower hind limb and hoof capsule. 1- Metatarsal bone; 2 - Fetlock joint; 3 - Sesamoid bone; 4 - P1 bone; 5 - Pastern joint; 6 - Tendons; 7 - P2 bone; 8 - Pedal joint; 9 - P3 bone; 10 - Corium; 11 - White line; 12 - Bulb sole region; 13. Sole; 14 - Hoof wall or horn; 15- Navicular bone.

Hoof Capsule

The hoof capsule is the base of support for all limbs attached to the P3 bone. It acts as the weight-bearing structure that transfers load to the bone. The growth rate of the hoof wall is approximately 5 mm per month, and the growth rate of the sole is approximately 3 mm per month (5, 13, 14, 16). The thickness of the sole is approximately 5-7 mm and the thickness of the keratin ventral to the pedal bone (and the apical toe) can be approximately 10 – 12 mm thick (5).

The corium is a vascularized dermal structure that supports the production of keratinocytes, which comprise the hoof structure (5, 13, 15, 17). There are four different regions of corium: perioplic, coronary, laminar and solar (13, 14, 17). Hoof horn material is produced through a process of proliferation and synthesis (15). Epidermal cells in the corium divide mitotically and proceed to grow outwards. Cellular differentiation occurs followed by programmed cell death. The outer layer is formed by keratin proteins from keratinocytes glued

together through intracellular cementing by the lining epidermal cells. This outer keratin material forms the structural hardness of the hoof and creates an impermeable barrier such that the interior is protected.

The white line is a region in the hoof capsule is located between the coronary wall horn and the sole. The white line region runs from the heel bulb around the abaxial wall to the toe tip, then back along the axial wall (5). Paetsch (5) took multiple measurements of the white line and found the approximate depth to be 4.72 mm in healthy hooves. When he was looking at animals with the toe tip necrosis syndrome, he found that the white line was much thinner, on average 3.74 mm thick. The white line region is made up of keratin material, made from terminal horn tubules. The keratin in the white line has a faster turnover rate and as such does not always have enough time to keratinize. The white line is also the meeting of two structures, this results in a material that is much softer that the other keratin materials, this can be seen in Figure 1-2 below (10, 12, 19).

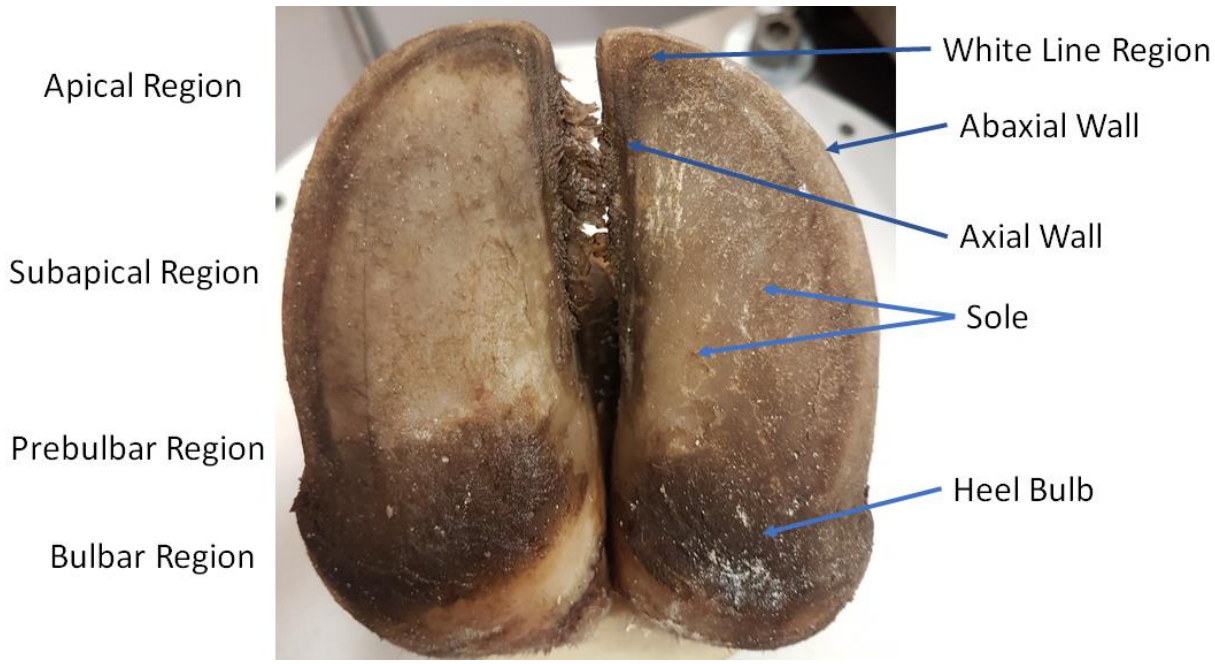

Figure 1-2 Sole of the hoof showing the different regions.

Weight Bearing/Biomechanics

The hind limbs bear approximately 40% of the weight of the bovine (5, 13). The lateral hoof bears more weight than the medial hoof. The lateral hoof is also slightly larger and has more weight bearing surface (5, 13, 14, 20).The heel strike refers to when the hoof first contacts the ground. Loading transfers from the heel to a flat contact, transferring load to the toe and

along the horn wall (5, 20). The sole does not make much contact with the ground due to the convex shape of the hoof (sole is curved away from the ground contact). The push off phase of gait has most of the body weight on the apical toe and white line region (20). Van Der Tol et al showed interesting results where the center of pressure was focused on the white line during the liftoff phase of the gait cycle, as well as the load reaching a higher level during this phase (20).

Hoof material properties are essential for the understanding of the way that load will affect the structure of a bovine hoof. Studies conducted on the mechanical properties of the horn wall suggests that the abaxial wall has a lower material stiffness than the dorsal wall (Elastic modulus E for the dorsal wall; 382 MPa, E for the abaxial wall: 261 MPa (9, 10, 12, 16, 21)). The sole and bulb have much lower material stiffnesses (E = 13.6 MPa (9, 10, 12, 16, 21)). The white line is defined as the meeting of these two materials, which creates an inherent weakness with a stiffness much less than the other areas. Collis et al tested the mechanical properties of the white line and found that the material stiffness was much less than the other structures of the hoof (E equal to 3.6 to 5.6 MPa (19)).

1.3 Disease

Lameness

Lameness is an abnormality of gait displayed by an animal, indicating that the animal is in some form of pain (2, 5, 8, 22-27). Changes to the normal gait and body posture can indicate a lame animal, as well as suggest a location of the problem (2, 5, 8, 22-27). Lameness can be identified by common indicators such as decreased physical activity, and reduced food, and water consumption (2, 5, 8, 22-27). There are many causes of lameness, so called “foot rot” is perhaps the most common, and is treated with antibiotics. Lameness left untreated many result in animal euthanasia. The feedlot industry is seriously concerned about lameness, both as an economic and animal welfare issue. The losses that are incurred by lameness include lower daily gain, treatment cost, and euthanized animal costs (5, 8).

Toe Tip Necrosis Syndrome

Toe Tip Necrosis syndrome (TTNS) is a disease that causes lameness in feedlot cattle. It is defined as a necrosis and/or toe abscess of the toe tip in the hind foot of cattle, and by separation of the apical white line (1-7). The soft connective tissue called the lamella is infected,

with necrosis spreading from soft tissue into the P3 bone. This disease can also lead to interphalangeal arthritis, and osteomyelitis of the middle (P2) and proximal phalanges (P1), flexor tendonitis, cellulitis and embolic spread of bacteria to the lungs, liver, and kidneys (1-7). In the Figure 1-3 below, a diseased hoof is shown with a healthy hoof for comparison.

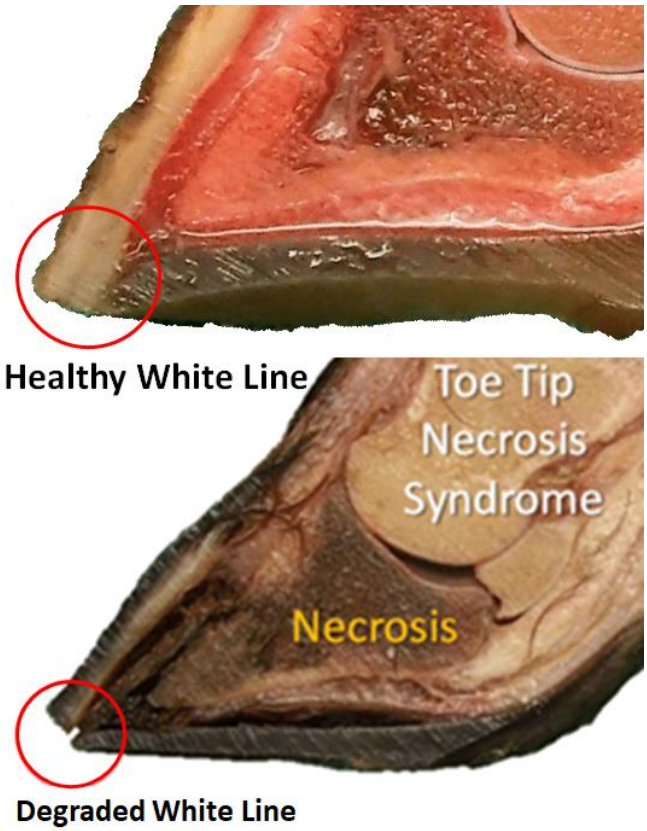

Figure 1-3 Top- Healthy hoof shown above with a normal intact white line. Tissues are healthy. Bottom- Diseased hoof shown below with degraded white line, severe necrosis is seen in the soft tissues, corium and P3 bone.

Confusion in the literature regarding the terminology of this disease causes misdiagnosis and leads to improper treatment of animals. Paetsch and Jelinski (7) proposed the term *toe tip necrosis syndrome* (TTNS) as a definition for the disease previously known as toe necrosis, toe abscesses, P3 osteitis, P3 necrosis, apicus necrotica, or apical pedal bone necrosis. The term TTNS is more encompassing definition, particularly when referring to feedlot cattle in Western Canada (3, 5-7). Some of this confusion may also have been with similar diseases affecting the hind limb, such as interdigital necro-bacillosis, laminitis, other soft tissue injuries (2, 5).

Diagnosis of this disease is made through physical examination and pinching of the cattle hooves with the use of a pinching tool (hoof testers), where a physical reaction helps in the diagnosis the disease. Diagnosis can also be made by nipping the apex of the toe and applying loading at the region of infection. Other methods of diagnosis are based upon visual examination, with veterinarians looking for clinical signs of lameness such as altered gait and animal expression of pain (2, 5, 8, 22-27). Early detection of this disease is very important due to the fast progression of the disease so that treatment can be administered; unfortunately, due to a lack of correct training and confusion regarding diagnosis in early stages of the disease, many animals are not diagnosed correctly.

Treatment of the disease is limited due to a lack of knowledge of this disease in feedlot cattle. The current practice is to treat the animal with antibiotics. Paring of the toe has also shown improvement; however, treatments do not always lead to full recovery (3-5). If the disease has progressed into the hoof capsule, amputation of the affected lower hind limb may be necessary. Though, in most instances, treatment consists of removing just the infected hoof capsule or digit. If the disease has progressed to the bone and further, this usually requires amputation of the affected area or euthanasia.

Apical White Line Separation

The initiatory first step of TTNS is thought to be white line separation, with the apical toe experiencing a separation of the horn from the sole material at the white line (1-7). While not clear in animals with early onset of the disease, this has been an observation in animals with severe cases of the disease. Figure 1-4 is an image of a hoof in which white line separation is evident. There is currently no explanation as to how or why the white line separates.

Figure 1-4 Image of the ground surface side of a bovine hind limb. This hoof has early stages of TTNS. The White line is separated; however, it is not clear and could go undiagnosed.

Gyan et al. (2) performed a study which attempted to predict TTNS based on apical white line separation. This study showed that practitioners were able to diagnose TTNS in 4/4 hooves (100%) based on white line separation, but also found that hooves diagnosed as healthy actually had the disease (2). This suggests that apical white line separation may be used to diagnose TTNS. However, marked separation needed for foreign material entry is not readily apparent upon visual examination of diseased hooves alone. Under physiologic loading, separation may be more predominant allowing for foreign material to enter the hoof. This study did not present any developing cases of TTNS; these hooves were taken from euthanized animals which had late stages of the disease. There is no evidence regarding how the apical white line separated.

Epidemiology

A few case studies have been published in the literature regarding the disease TTNS (5-7, 26, 28-34), but they provide limited information on the epidemiology and etiology; they were also published under a different descriptor of the disease. Paetsch (5) presented the epidemiology of the disease with a focus on Western Canadian feedlots. TTNS was present in 19 of 48 feedlots, and was present in 3.8% of lots. It was found that the disease was prone to clustering (5). Specifically, lots either had many cases of TTNS or none. In feedlots with the disease is was

found on average to be present in 1.22% of load lots (5). This is most likely due to the environmental conditions that animals are subjected to. As such, the cause of TTNS and white line separation is probably partly environmental, possibly due to facility design, nutrition, or animal handling. As well, animals exhibit behavioral similarities in feedlot facilities (e.g. irritable, unruly, and aggressive) (2, 3, 5, 6).

In terms of background characteristics, most of the animals that had the disease came from auction (77%)(5). Other sources include back-grounded, pasture, and ranch direct. The infected animals were either yearlings or calves (55% of cases being yearlings; 45% being calves) (5). Paetsch speculated that more yearlings were afflicted due to the larger weight (5). The time from entering the feedlot to treatment in animals that were treated was between 13 and 25 days (5). Given that infection takes a similar time to manifest and present (5), this puts the initiation of infection near feedlot arrival or shortly afterward. This disease has therefore been reported to be an arrival or transport related disease (3, 5, 29-31, 33).

Bacteria present

Paetsch conducted a post-mortem study of the tissues of the bovine hoof to try and identify possible causes of the disease (5). This study included sectioned bovine hind hooves, soft tissues of the bovine hind limb as well as hoof material. Findings showed that there were multiple bacterial pathogens present. A large percentage of pure isolates (75%) were attributed to Escherichia *coli*, Streptococcus, Trueperella *pyogenes*, and Fusobacterium *necrophorum*. These bacteria (which were found to be possible risk factors) were found to have an association with TTNS. Paetsch also found that TTNS case animals were 3.8 times more likely to have bovine viral diarrhea virus (BVDV) (5). The role of this virus in TTNS is still unknown but was speculated to have disruption effects on the corium, resulting in a weaker material. BVDV is also known to cause immunosuppression, which would allow bacteria populations that are associated to the disease to spread more rapidly (5, 35). The bacteria are typically found in manure located in feedlots where animals would be walking. The bacteria are also found in the chute, where animals could be exposed to agitation.

Leading Hypotheses

There are three prevailing theories regarding the possible cause of TTNS. The first theory deals with feedlot cattle being transported in trailers, which can involve long standing periods with little ability to move. With excess standing, hypostasis is thought to occur (settling of blood in the hoof), resulting in ischemic necrosis of the apex of the P3, followed by destruction of the apical white line (1-7).

The second prevailing hypothesis suggests that cattle have an adjusted diet before transport and after arrival at the feedlot, whereby metabolic disturbances cause a weakness in the white line. This weakness, coupled with laminitis, could allow for displacement or rotation of the P3 relative to the hoof capsule, leading to the P3 bone pushing the sole apart from the white line leading to infection (1-7).

The third and prevailing hypothesis based on observations by Paetsch, Gyan et al. and Jelinski et al., (2, 3, 5) suggests that mechanical damage coupled with loading is the cause of white line separation. Specifically, mechanical damage in the form of excessive wear on the sole caused by contact with abrasive surfaces such as concrete, metal, and ice. Mechanical damage is even more plausible considering the chute systems of feedlots which contain abrasive surfaces. Aggressive behaviour in the chute has also been observed, with situations of extreme loading (e.g. pushing forward into the animal in front, stamping (3)). This loading, combined with excessive wear is thought to cause the white line to separate (1-7). This separation then leads to a bacterial infection spreading from the apical white line to the P3 bone. This third hypothesis is the focus of this thesis.

1.4 Summary

Current literature provides some insights into TTNS. The disease is a major problem in Western Canadian feedlots, causing lameness. The current literature includes case studies, epidemiology, treatment, microbiology, and has pointed to multiple hypotheses regarding the cause of the disease. The prevailing theory of mechanical damage, reported in many of the studies warrants further investigation.

Chapter 2: Research Questions and Objectives

2.1 Research Question

The proposed research will attempt to answer the question: Does mechanical damage and loading separate the white line, leading to an opening where debris and bacteria could enter the hoof?

2.2 Objectives

To address this overall research question, the objectives of this research are as follows:

1. Determine whether white line separation is associated with load.
2. Determine whether white line separation is associated with mechanical damage (due to wear).

2.3 Scope

Chapter 3 describes the investigation of white line separation in healthy and diseased hooves. The hooves are loaded static compression to determine the area of white line separation. Chapter 4 outlines research, testing specimens under cyclic fatigue loading with and without mechanically induced damage to determine if white line separation develops in specimens with wear or not under mechanical loading. Chapter 5 discusses the research completed and how it fits into the current literature. Chapter 5 also outlines recommendations for future research.

Chapter 3: Effect of Compressive Loading on White Line Separation

3.1 Synopsis

Healthy bovine and bovine hooves affected with TTNS were obtained and tested using a mechanical testing apparatus which applies static compressive loading while an underside camera captures white line deformation in the form of two-dimensional (2D) separation area. Outcomes from this objective include: the loading parameters (compression) that lead to separation of the white line, and the area of the white line separation. Bovine hooves were also imaged with a high resolution peripheral quantitative computed tomography (HR-pQCT) scanner and a contrast agent to visualize three-dimensional (3D) white line separation in the form of separation volume. This work provides insight regarding loading conditions leading to white line separation.

3.2 Introduction

The prevailing hypothesis regarding the cause of TTNS is mechanical damage (in the form of excessive loading and abrasion to the hoof) leading to separation of the white line and pathway formation for bacteria entering the hoof (1-7). The objective of this *ex vivo* study was to determine the effect of loading on white line separation to assess the merit of this potential initiatory cause of TTNS. The hypothesis is that white line separation increases with increasing load.

3.3 Materials and Methods

Specimens

Specimens of yearlings were acquired (14 diseased and 10 healthy bovine hooves) from participating veterinary feedlots (36, 37). TTNS was diagnosed by participating veterinarians. Specimens were sectioned at the fetlock joint from the hind limb of the animal. These specimens were frozen and shipped to us. Freezing specimens has been shown to have no effect on the strength or stiffness of the bovine hoof (38). However, specimens were thawed for testing as soon as possible on shipment receival. Eleven specimens were found to have TTNS while three animals were found to have died from a hoof infection not related to the disease (further

illustrating the need for better naming, understanding and diagnostic tools). Healthy controls were collected from animals having died from other means.

Specimens were potted (secured) in an anatomical position for testing. This was done by dissecting the specimen down to the P1 bone; all soft tissues were removed from the P1 bone. Polymethylmethacrylate (PMMA) was used to fix the hooves in an anatomical position. A hacksaw was used to remove material above the fixation. The hoof was then potted in Denstone (Heraeus Kulzer Inc.), as shown below (Fig. 3-1). This potting procedure allowed the testing of specimens in the mechanical testing apparatus. The complete procedure can be found in appendix A.

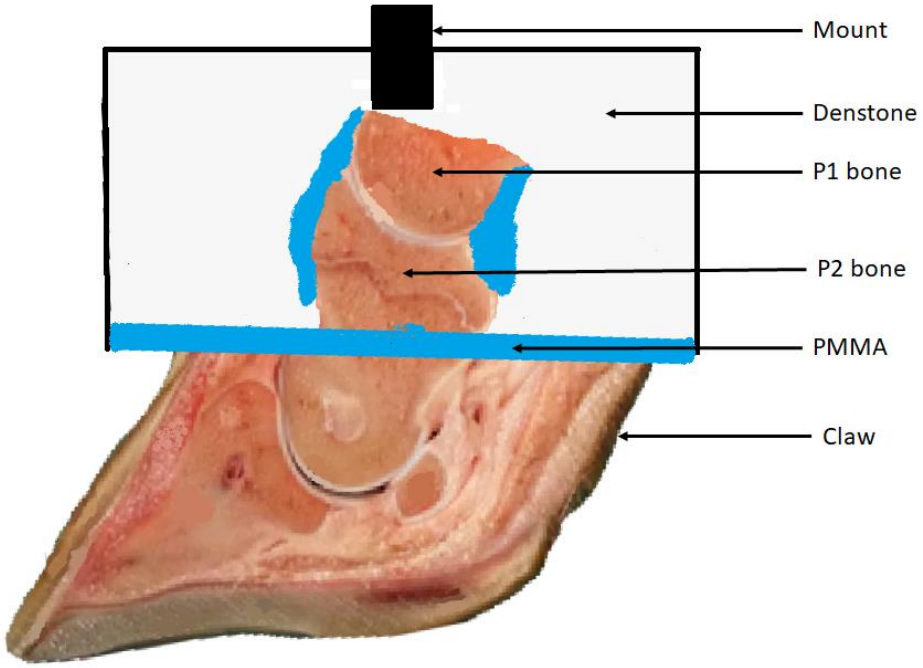

Figure 3-1 Fixated Hoof in Denstone and PMMA

Mechanical Testing and 2D Imaging

A custom testing apparatus was integrated with a servo-hydraulic material testing system (MTS Bionix), as shown in Figure 3-2. The apparatus was designed such that loading could be applied to the hoof specimen while a camera (Point Grey Chameleon3 5MP monochrome camera) located at the bottom of the camera housing, 305 mm (12 inches) underneath the hoof,

took images of white line separation. The hoof and camera were separated by an acrylic plate, as shown below (Fig 3-3).

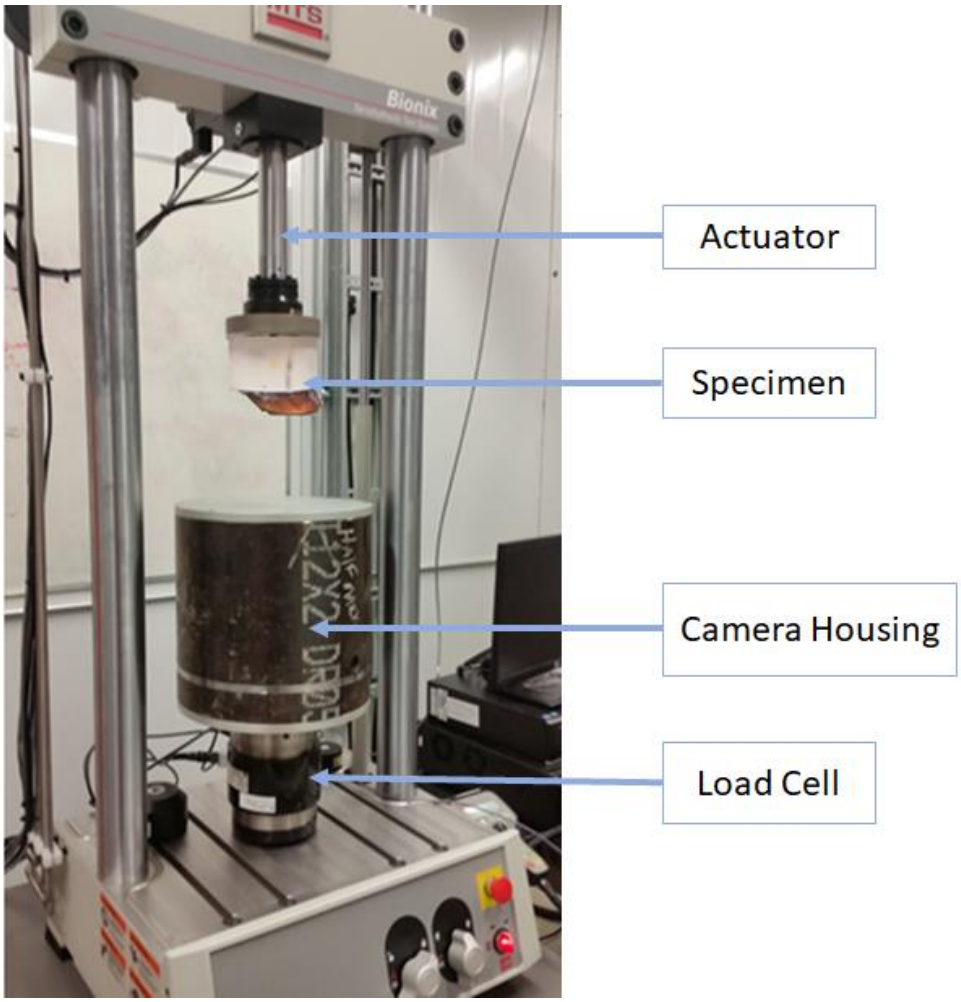

Figure 3-2 Mechanical Testing apparatus for loading. This apparatus has a housing for the camera. On top is the loading plate which can be switched to acrylic for imaging, and aluminum for fatigue loading (discussed in Chapter 4).

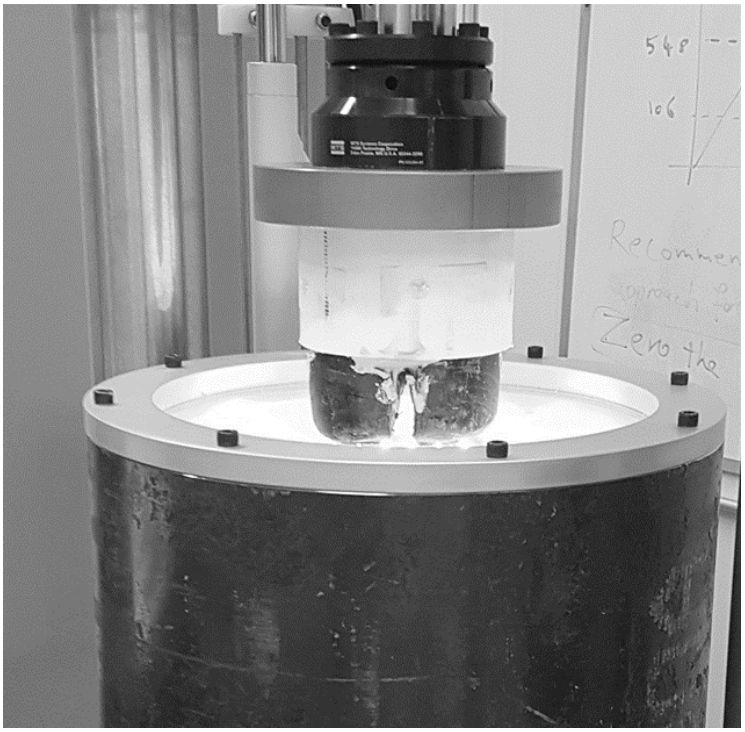

Figure 3-3 Mechanical testing apparatus setup showing the acrylic plate. Base is shown as the large tube on the bottom. A camera is located inside the tube which takes images upwards. The MTS Bionix applies compressive load to the hoof downwards onto an acrylic plate. The specimen is shown potted in an anatomical position.

Loading was applied to the hooves to capture white line separation under load. Images were taken at the different loading conditions. The first load was 1 kN which was representative of body weight. An extreme loading situation was then applied (2 - 3.5kN) which would be found when the animal is aggravated and pushing in the chute, or shuffling during transport. Static compressive loading was applied at 2 kN and increased by 0.5 kN for each loading step to track white line separation with increasing load (9). Testing was stopped if the hoof was too damaged to continue. Many diseased hooves were able to withstand 3.5 kN, which was then used as the limit for loading on healthy hooves. A dental puncture tool was used to verify separation by probing visualized separation area.

3D Imaging

An HR-pQCT scan was taken of the specimens at a voxel size of 41 µm. The volume of interest (VOI) was four blocks, each with a height of 9.02 mm. An image enhancing contrast agent (Cyto II Conroy(Iodine)) was used to show white line separation space as initial images without contrast agent did not provide enough detail. To visualize white line separation, a suction pump and a contrast agent bath were used to pull the agent up into separation spaces. A hole was drilled into the horn wall 1.5 cm above the ground contact surface for the suction fitting. Suction was applied for three mins at a pressure of -20 psi. Shown below (Fig. 3-4) is a setup of the contrast pump and contrast agent bath.

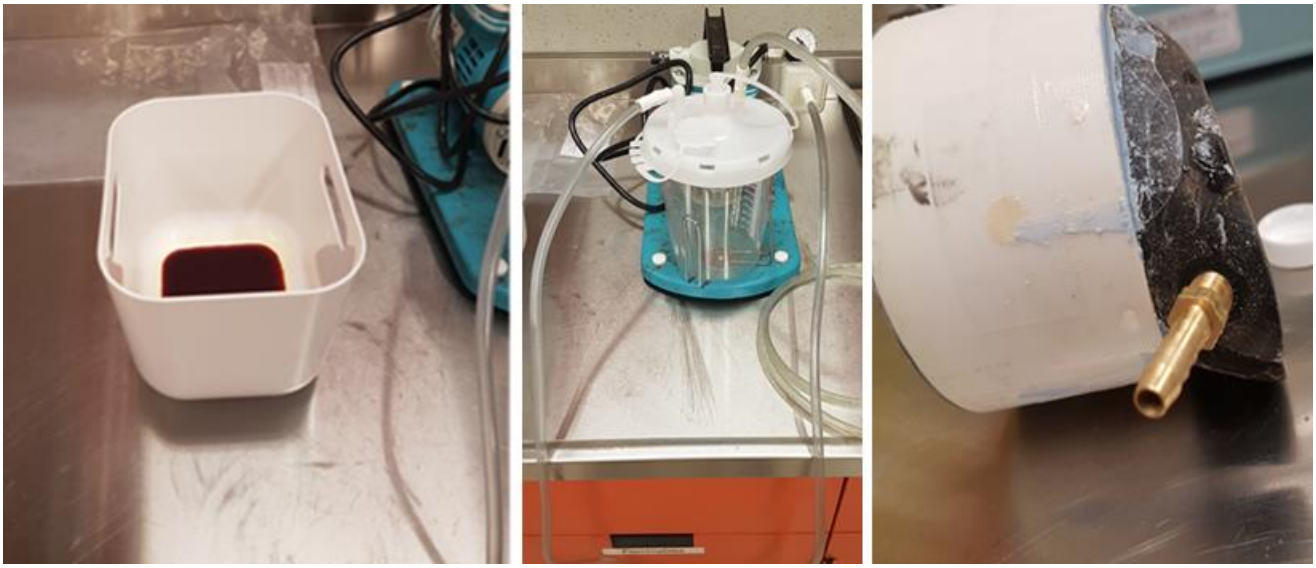

Figure 3-4 Left – Iodine contrast agent bath. Middle - Contrast agent pump, Right – Hoof specimen with pump attachment

Image processing

Two dimensional and 3D image volumes were segmented to show separation of the white line. Images were loaded into a commercially available software (Analyze 10, Analyze Direct Inc. Overland Park, KS). Image processing software allowed us to segment white line space using a region growing technique to quantify separation area (with 2D images) and volume (with 3D images), as shown below (Fig. 3-5 and Fig. 3-6). Manual segmentation with a tablet and a stylus (WACOM, Portland Or. U.S.A.) was used to correct segmentation errors (39). These segmentations were then imported to Matlab (The Mathworks Inc., Natick, Massachusetts,

U.S.A.) to calculate the area and volume. For 2D images, a ruler was used for calibration to determine the actual area of each pixel. Calibration indicated 19.7 pixels per millimeter in both the x and y directions. With imaging through mediums there is inherent warping. A calibration was performed with grid paper to show the (fisheye) distortion of the images. The distortion was found to be 3 pixels across the entire image, which had very minimal effects on white line separation as the hooves were centered in the images. Measurement precision errors were accessed by segmenting each image three times with error characterized using root mean square coefficients of variation (CV%). The CV% precision error was calculated to be 7.8% for 2D segmentations. The 3D scans were segmented using a morphological thresholding technique in Analyze 10. The specific threshold intensity was found by plotting a line profile over the hoof and finding the maximum intensity of the hoof material. This technique allowed for the hoof material to be removed and only contrast agent inside the hoof to remain in the volume. The threshold that was used was an intensity of 1350 HA.

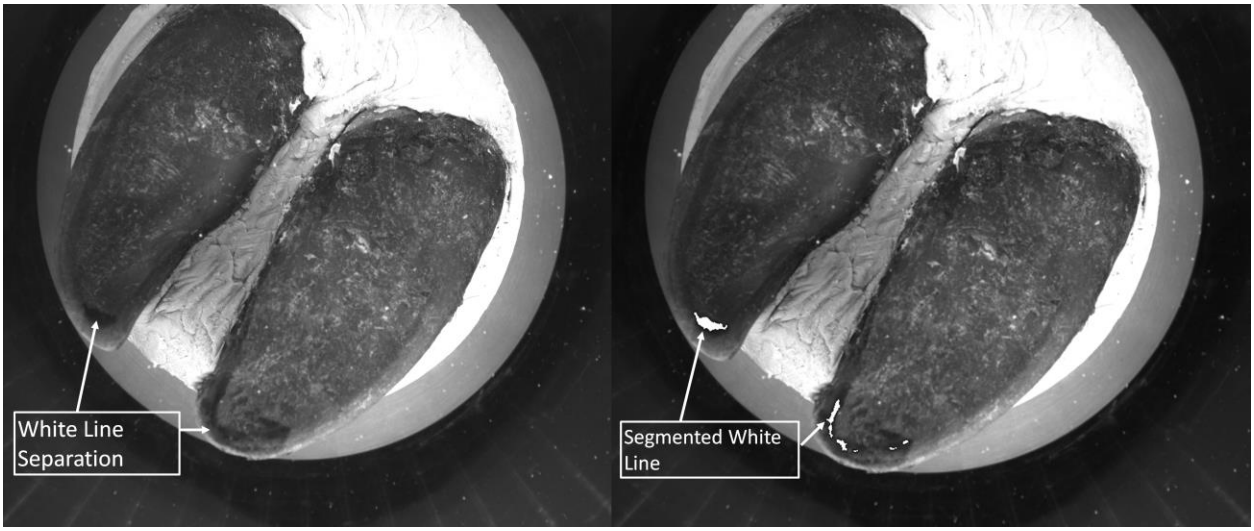

Figure 3-5 Segmented white line separation: Left- is specimen L at 1 kN without segmentation, Right- Same image (specimen L at 1 kN) with object map overlaid on top of the image. This segmentation was done using a region growing technique. Manual correction was used to fix errors with region growing segmentation.

Figure 3-6 Sagittal slice of healthy and diseased bovine hoof. This hoof has had contrast agent pulled up into it via the contrast agent pump. The contrast agent has accessed the hoof and sits in the spacing.

Statistics

Factorial repeated measures ANOVA was used to assess the association between load and white line separation area. Linear regression was used to assess trends in the data and determine if there was an overall association between load and separation area. Student's t-test was used to compare white line separation area between the diseased hooves and the healthy controls at the different loading levels. The Student's t-test was also used to compare white line separation volume. The statistical output is contained in the appendices.

3.4 Results

Repeated measures ANOVA indicated an association between load and separation area ($F=26.04$, $p=0.012$) in diseased hooves. For healthy hooves, no association was found between load and separation area ($F=3.032$, $p=0.109$). Factorial repeated measures ANOVA found that diseased hooves showed significantly more white line separation than the healthy hooves (Pillai's trace value 0.953,$p<0.001$). The pairwise comparison also indicated a significant difference between white line separation area at all loading levels in the diseased hooves versus healthy hooves. Regression analysis indicated a linear association between load and separation

area for diseased hooves (R^2 = 0.76, p<0.001) but no association was noted for healthy hooves (R^2= 0.097, p=0.028). Data from the segmentations are shown in the two plots below (Fig. 3-7).

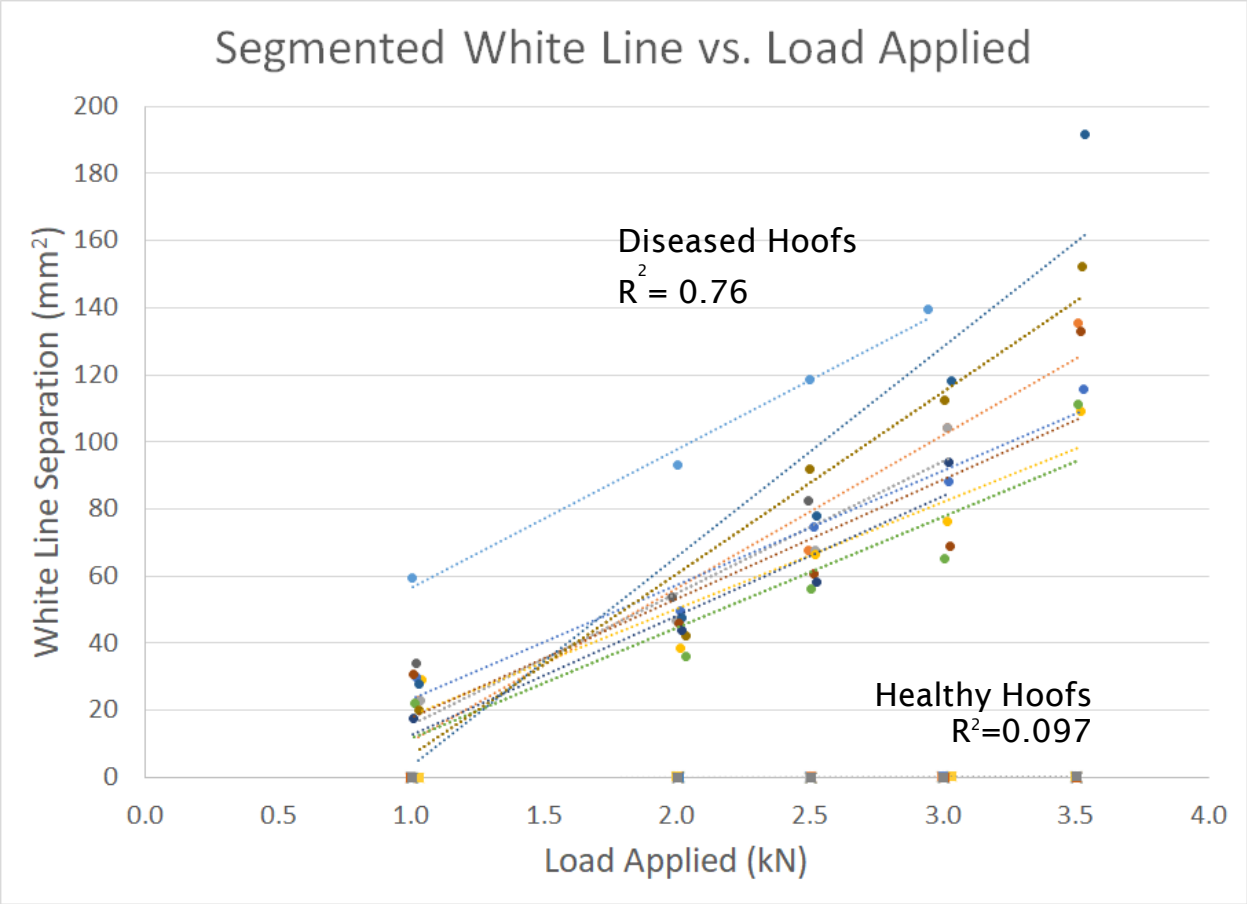

Figure 3-7 Plot of white line area versus applied load. All diseased hooves showed increasing trends while healthy controls remained at approximately zero. The R^2 value for diseased hooves was 0.76 while the R^2 value of the healthy controls was 0.097

The Student's t-tests indicated a significant difference between white line separation area in the diseased hooves and the healthy controls at each of the loading levels (p<0.001, Table 3-1). The Student's t-tests also indicated a significant difference between the diseased and healthy hoof separation volume (Mean ±SD volume: Diseased = 304.5 ± 256.7 mm^3, Healthy= 0.117 ± 0.170 mm^3 p=0.002). Of note, one diseased hoof was excluded from this analysis due to a large build up in contrast agent whereby the disease created a pocket of necrosis in this specimen which held onto the contrast agent more than the other specimens.

Table 1-1 Summary of Students T-tests comparing white line separation in diseased and healthy hooves in 2D images at different loading levels

Mean White Line Separation Area(mm^2)			
Load (kN)	Diseased Hooves	Healthy Hooves	P-value
1.0	29.12 ±12.11	0.02 ±0.04	<0.001
2.0	52.24 ±16.07	0.06 ±0.08	<0.001
2.5	77.84 ±18.48	0.04 ±0.08	<0.001
3.0	101.26 ±24.74	0.10 ±0.11	<0.001
3.5	139.67 ±30.15	0.10 ±0.11	<0.001

3.5 Discussion

Study results indicated a positive association between white line separation and load for diseased hooves whereas no association was found with healthy hooves. This study also noted more separation in diseased hooves than in healthy hooves at all loading levels. The healthy hooves showed no separation.

Separation found with diseased hooves under normal (1 kN) and extreme (3.5 kN) loading indicate that loading could contribute to TTNS. The large separation area found in diseased hooves at extreme loading conditions suggest that animals who experience this loading condition may be more likely to have a large separation of white line, leading to a faster onset of TTNS. Given that there was no evidence of separation in the healthy controls, this finding suggests that static compressive loading and isolated excess loading does not directly lead to TTNS. Rather, loading likely exsacerbates and accelerates the disease process.

Of note, separation of the white line was only seen under loading. When hooves were unloaded, observations of the white line separation were minimal. The puncture tool used to verify separation, penetration of the hoof in the observed locations of separation on the image was done; however, area previously seen (under loading) was not as apparent. This leads to the hypothesis that, once the white line is broken down and separation occurs, material and bacteria may enter the hoof and be held there due to the large separation closing. This collection of debris and bacteria in the hoof creates ideal conditions for bacterial growth, leading to rapid progression of the disease. Observations of the diseased hooves in this study showed similar trends to what Paetsch and Jelinski found (3, 5). Specifically, diseased hooves were visibly worn down, and the entry means for bacteria to penetrate the unloaded hoof were not readily apparent. However, it was possible to visualize white line separation upon specimen loading.

HR-pQCT scanning showed that the contrast agent was able to penetrate the hoof structure in the diseased hooves, but not the healthy hooves. These results indicate that bacteria are able to enter the hoof capsule, further supporting the hypothesis that the white line separation is a precursor to TTNS. Methods used in 2D imaging and CT imaging could potentially be used as a clinical diagnostic tool for capturing early stages of TTNS.

3.6 Conclusion

This study found a positive association between white line separation and load in diseased hooves but no association in healthy hooves. There was more separation in diseased hooves with increased loading levels. Separation volume was also higher in diseased hooves. Given that no separation was found with healthy hooves, these results suggest that pure compressive loading, even at excessive levels, is not an initiatory aspect in the disease. Whether wear or fatigue loading (another form of damage) is related to white line separation has yet to be determined. These findings support the hypothesis that mechanical loading is involved in TTNS etiology or disease exacerbation and acceleration. These results suggest that white line separation is a feasible means of entry for bacteria, potentially leading to TTNS.

Chapter 4: Effect of Mechanical Damage on White Line Separation of Bovine Hooves

4.1 Synopsis

Ten paired bovine hooves (10 healthy, 10 manually damaged) were tested under fatigue loading for 36,000 cycles, with impact loading included at multiple time points to simulate extreme loading (9, 40, 41). Damaged hooves had 6-8 mm of hoof material removed via rasping, which simulated bovine dragging when agitated, wearing down the hoof. Specimens were imaged with the underside camera discussed in Chapter 3. This process was repeated five times during cyclic testing to measure separation area. Bovine hooves (post-fatigue loading) were imaged in the HR-pQCT with contrast agent to measure separation volume.

4.2 Introduction

The cause of the disease TTNS is currently unknown. Previous work suggests that mechanical damage to the bovine hoof leading to white line separation and bacterial penetration is the initiatory cause (1-7). The previous chapter presented results showing that the white line of diseased bovine hooves separates under loading, but healthy control hooves did not.

It was undetermined if separation will occur in a specimen subjected to mechanical damage in the form of fatigue loading and wear. Fatigue loading is the weakening of material through loading applied over many cycles. Fatigue loading will be employed in this study as a means of break down of the white line. Fatigue loading has not been previously done to bovine hooves. Fatigue loading will be used to mimic normal loading in the feedlot in the first week on arrival. The objective of this research is to determine whether mechanically induced wear, with fatigue loading is associated with white line separation.

4.3 Materials and Methods

Specimens

Ten pairs of healthy hind limbs were collected from cattle that were euthanized for means other than hind limb conditions. The lower limb was prepared in the same manner as outlined in Chapter 3. Ten specimens were healthy controls and the remaining ten pairs had mechanically damage induced. This damage represented a hoof that has been worn down on surfaces in the

feedlot via rasping. Damage was induced via a veterinary hand file by rasping down the hoof based on reported thinning of the sole (5). Hooves were rasped down between 6 – 8 mm. A 3D digitizer was used to identify the surface of the hoof pre- and post- rasping. Surface points were fit to a plane (Matlab), with the difference in plane heights defined the level of rasping. The figure (Fig. 4-1) below shows a hoof that has been rasped. This amount of material was removed near the thickness of the hoof sole, so discretion was used to avoid filing away all of the keratin. Pre- and Post measurements were done with a ruler to help perform a uniform removal of material.

Figure 4-1 Image showing a hoof that has had mechanical damage induced with a veterinary file. The file was used to rasp down hoof material.

Fatigue loading and 2D Imaging

This study used a similar mechanical testing apparatus to the system used previously in Chapter 3. A modification was made so that the loading platform also had an easily interchangeable aluminum plate for fatigue loading. The testing apparatus was designed to endure long cyclic loading periods then easily change to the acrylic plate to take an image.

Fatigue loading was applied to the bovine hooves. The loading regime is shown below (Fig. 4-2). This shows that the loading cycled between 100 N and 1000 N at a rate of 2 Hz for 7200 cycles (approximately one-hour). This loading regime is a better representation of what a normal hoof would be experiencing than static compressive loading that was used in the previous study. This loading represented normal body weight of an animal placed on a hind limb during walking. Body weight was calculated based on average weight obtained from previous work (bodyweights for individual specimens were not available) then calculating the weight that would be applied to a single hind hoof using the suggested weight distribution from literature of 40% body weight on the hind limbs. Impact loads were intermittently applied which went to 3000 N at a rate of 6000 N/s to represent the possibility of extreme loading levels for the bovine in the chute, or stamping and single leg standing that could be experienced during travel. This impact load represents an ~3x body weight scenario. After the first loading was completed, an image of the bottom of the hoof was taken. This process was repeated five times, though the total number of cycles was limited to 36,000 due to the capabilities of the operator and shared access of the testing system. The 36,000 cycles represent an approximation of 7 days on the feedlot which is representative of the disease timeline (3, 5).

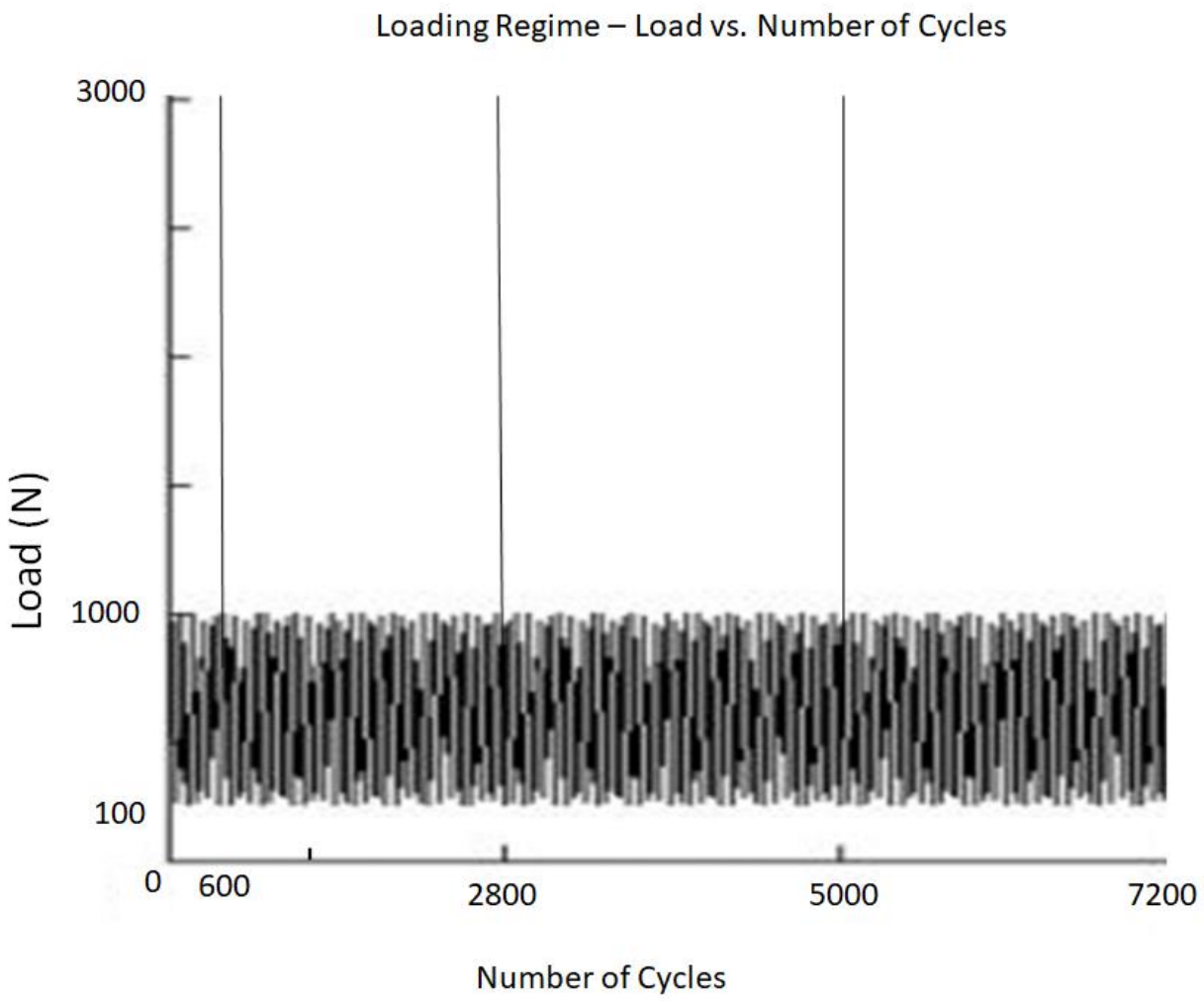

Figure 4-2 Representation of the fatigue loading regime. There are three instances of impact loading, one near the beginning at 600 cycles, then two more at even intervals through the loading regime.

Images were taken initially at and after every 7200 cycles under loading (1kN) using the camera (Point Grey Chameleon3 5MP camera). This allowed us to track progression of separation with time. Images were segmented using the same method as outlined in Chapter 3. Measurement precision errors for 2D images were accessed by segmenting each image three times with error characterized using CV%. The CV% precision error was calculated to be 6.0%.

3D Imaging

Post-testing, HR-pQCT images were aquired of the damaged and healthy hooves in the same manner as outlined in Chapter 3. The first 3D scan was performed to confirm the healthy diagnosis of the hooves. The second 3D scan was performed with a contrast agent (Cyto II Conroy (Iodine)) as described in Chapter 3.

Statistics

Factorial repeated measures ANOVA was used to access the association between time (in cycles) and white line separation area. Paired t-tests were used to compare the results from the damaged and the healthy control hooves at different time points. Paired T-tests were also used to compare segmentation volumes. The statistical analysis is shown in the appendices.

4.4 Results

Factorial repeated measures ANOVA of the fatigue testing showed no association between healthy and damaged hooves when looking at separation area at increasing cycle counts (Pillia's trace value = 0.233 p=0.376). Repeated measures ANOVA found no trends in the data (Healthy Pillai's Trace=0.630 p=0.109; Damaged Pillai's Trace=0.496 p=0.319). Pair wise comparisons also showed almost no differences between the healthy or damaged hooves at the different cycle counts (Table 4-1). At the 14400-cycle count, significant differences were found. However, this result shows that there is greater separation in the healthy controls than there is in the Damaged hooves. The results indicate that there is no change in the amount of white line separation with fatigue loading, with or without damage.

Table 4-1 Summary of Student's t-tests comparing 2D white line separation at different cycle counts

Mean White Line Separation Area (mm^2) at Fatigue Loading Cycle Counts			
Cycles	Damaged Hooves	Healthy Hooves	P-value
7200	0.01±0.05	0.06±0.09	0.12
14400	0.01±0.03	0.12±0.12	0.03
21600	0.02±0.05	0.13±0.16	0.11
28800	0.03±0.06	0.10±0.14	0.08
36000	0.07±0.08	0.15±0.18	0.29

Paired tests of contrast enhanced HR-pQCT scanned volume indicated no significant differences between the healthy controls and the diseased hooves (Mean ±SD volume: Damaged = 0.12 ± 0.17 mm^3, Healthy=0.14 ± 0.25 mm^3; p=0.90). Shown below in Figure 4-3 are sagittal sections from HR-pQCT scans of a healthy and damaged hoof. These show that no contrast agent penetrated the white line. A diseased sagittal section is shown for comparison.

Figure 4-3 Sagittal sections of HR-pQCT scan volumes at the apical toe. Healthy and damaged hooves show no contrast agent penetrating the white line. A diseased hoof is shown for comparison from the previous study.

4.5 Discussion

This study found no association between cycle count and separation area for both the damaged and healthy hooves. Also, there were no differences in separation area between damaged and healthy hooves at all time points except 14400 cycles. At the 14400 cycle time point there was a statistical significance showing that healthy hooves have more white line separation than the diseased. This can be explained as error. There was no difference in separation volume post fatigue loading. As such these results suggest that artificial damage and fatigue loading at the tested quantities may not lead to separation of the white line. It is possible that a higher degree of artificial damage and fatigue loading at larger loading magnitude or increased testing duration may have led to white line separation.

Although study results indicated no white line separation, this study provided some insight. Following material removal, the white line was much softer, as observed by manually applying force with a dental puncture tool. This observation could provide the means for uncovering the cause of white line separation. We suggest that the stimulus could be a puncture, shear loading, or possibly bacteria causing the white line to breakdown following abrasion to the hoof. A puncture from a rock from broken concrete is feasible considering the soft keratin material and the feedlot structure. Shear loading is another loading scenario that bovine could face in the feedlot or during transport. After the keratin material was removed through mechanical damage, there was a soft thin layer remaining. It is possible that the bacteria present in a feedlot scenario could digest this softer layer of keratin. Once inside, this bacteria could develop the infection causing the disease.

HR-pQCT scans were employed to visualize white line separation in the form of separation volume. When compared against separation volume of diseased hooves from Chapter 3 (304.56 mm^3), it is clear that little separation occurs with fatigue loading, with (0.12 mm^3) and without damage (0.14 mm^3). It was worth noting that some contrast agent appeared on the outside of the hoof that was captured in the scans (this explains the small amount of separation volume). Ringing artifacts were also present in the 3D images, which are common errors that occur in CT scans. Thresholding removed most of these artifacts; however, ringing could explain observed segmentation volume in healthy and damaged hooves.

4.6 Conclusion

This study found no association between cycle count and separation area for both the damaged and healthy hooves. Also, separation area and volume were small following fatigue loading with and without wear. These results suggest that white line separation (and subsequent TTNS) is not due to mechanical damage induced by wear or prolonged repetitive loading. Our results indicate that another loading parameter or a biological event initiates TTNS.

Chapter 5: Discussion

5.1 Overview of Findings

Currently, it is unknown what causes white line to break down and separate in bovine hooves. Research on this disease is limited, with only a few epidemiological and case studies (1-7, 14, 24, 26, 28-33). The most prevalent hypothesis is that mechanical loading and damage to the hoof cause white line separation. In this research, we developed a method to test bovine hooves under load while being able to visualize the white line. This proved to be an effective tool for quantifying white line separation and could be used in future *in vivo* studies to understand the disease better (e.g. capture white line separation while bovine walk over a platform and camera). This research showed that diseased hooves had substantial white line separation when placed under load; however, when healthy hooves and artificially damaged specimens were placed under load, little to no separation was noted. The findings from this research indicate that mechanical loading and damage are not solely responsible for the separation of the white line but extreme loading likely exacerbates and accelerates TTNS. These results indicate that there is another factor that needs to be present for white line separation.

5.2 Comparison to Existing Findings

There is little literature on the mechanical testing of bovine hooves. Previous mechanical testing research focused on determining material properties (9-12, 19, 21), not investigating white line separation. This study is novel in that it investigates the hypothesis that mechanical loading and damage causes white line separation. Results from this study disagree with suggestions in the literature citing mechanical damage and loading being the lone cause of white line separation and eventual TTNS. This research suggests that mechanical loading plays some part in disease etiology, specifically in that extreme loading likely accelerates disease progression by increasing separation, thereby creating a pathway for bacteria. The findings from Chapter 3 support what was found by Gyan et al (2), in that apical white line separation is the likely entry point for bacteria.

Observations from Chapter 4 suggest other possible means may be the cause of white line separation, where loading only aids in this pathway formation. There are other hypotheses presented in the literature about hypostasis and dietary changes, which coupled with mechanical loading may lead to separation of the white line (1-7). Thinning of the white line material as

observed by Paetsch was recreated by artificial damage (5). When this was done it was also observed that there is softer keratin material underneath. This weaker white line could be more susceptible to other types of loading such as shear and puncture loading. This softened white line may be more at risk from bacteria, as bacteria might be able to digest the softer keratin material and form a pathway into the hoof.

5.3 Strengths and Limitations

This research has various strengths that were not discussed entirely in previous sections. The first main strength of this research is the development of a useful tool for characterizing white line area. This tool will allow researchers to better understand TTNS, provide useful insight for future research, and even possibly be used to diagnose TTNS in the field. Specifically, this tool could be implanted in the floors of feedlots, or in a portable, low-profile platform for farmers to monitor hoof health. This tool may also help in the diagnosis of other hoof related diseases. The second strength of this thesis research is that it is the first to mechanically test bovine hooves in a situation similar to *in vivo* loading conditions. This could become a standard testing method for testing bovine hooves in future research. This testing method could also apply to other ruminants that need to have images taken of the underside of their feet.

Limitations of this research pertain to sample size, samples, loading magnitude, and loading configuration. First, the sample size was small (~10/group). The sample size was limited as specimen testing was quite long (~1 day/specimen). There was also an economic downturn in the feedlot market in Canada and obtaining TTNS specimens was difficult as feedlots saw lower numbers and therefore fewer animals that were infected with the disease. In Chapter 4, paired specimens were used to account for the small sample size. Given the small variability (in terms of SD) noted with healthy and mechanically worn hooves, it is doubtful that we were underpowered to detect a significant difference in separation area and volume. Second, we were limited to the specimens that were sent to us, this meant that the specimens were animals with no TTNS and later stage TTNS. Although we suggest that loading is involved in white line separation and TTNS, it is unclear if separation occurs at early stages of the disease. Future research should strive to test healthy as well as early stage TTNS specimens. Third, for fatigue loading we only tested specimens for 36,000 cycles with loading of a magnitude 0.1 kN – 1.0 kN. Longer testing with a higher load range and magnitude may have led to separation. In terms

of loading it would have also been better to use pressure as our measurement instead of load, accounting for contact area of the hoof, this would eliminate some of the variability naturally found in bovine hooves. Fourth, in this research we only assessed compressive loading. It is possible that hoof dragging may increase separation by placing high tensile loads on the hoof. These are directions for future research.

5.4 Conclusions

1. These findings support the hypothesis that mechanical loading is involved in TTNS etiology through disease exacerbation and acceleration.
2. These results suggest that white line separation (and subsequent TTNS) is not due solely to wear or prolonged repetitive loading. Our results indicate that another loading parameter or a biological event initiates TTNS.

5.5 Clinical Significance

The effects of this research in the field could lead to change the direction of research into TTNS. Specifically, these results suggest that regular mechanical loading and damage induced artificially may not lead to the disease TTNS. These findings could help point to a more multifaceted disease perspective, suggesting that there is much more to the break down of the white line and give more grounding to other hypothesis such as dietary changes and hypostasis. These results suggest that current practices in the feedlot may not be what is causing the disease. This could have huge financial implications for feedlots, as there may not be a need to change current practices, saving large amounts of moneyf. The tools and methods developed in this study could also be utilized in commercial feedlots to help with the diagnosis of the disease and in future studies of other hoof issues. This study will also raise awareness of TTNS, bringing attention to the disease and associated negative effects.

5.6 Recommendations for Future Research

Next steps for research into TTNS should be:

- Begin testing the hooves in a shear loading scenario in which there is also a horizontal loading component.
- Investigate the softer keratin which was uncovered when the damage was induced to the hoof through a puncture or indentation test.
- There is the possibility that the softer keratin is vulnerable to bacteria that can break down keratin. This could be another possible direction to uncovering how the white line separates.
- It is possible that separation was occurring with fatigue loading but was missed due to poor image resolution. It would be beneficial to image samples of tested hooves with standard micro-CT or synchrotron micro-CT.

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Appendix

A. Sample Preparation Methods

Sample preparation methods followed the standard operating procedure for the laboratory in Engineering Building(2C50), University of Saskatchewan, titled: Wet Preparation of Bovine Hooves. The following is an abbreviated version of the standard operating procedure. The full version is available upon request from the lab.

1. Donn the proper PPE.

2. Get out the necessary dissection and sample preparation equipment. Below is a figure showing the surgical tray that you will first remove from the orange overhead shelf. In this part of the lab, we assume that everything is contaminated (regardless of whether surfaces and tools are clean). Therefore, ensure you are wearing CLEAN gloves when preparing dissection space and getting equipment

   Line this tray with a paper towel. If there is not enough paper towel on the shelf, get some more from the storage shelves on the other side of the lab. This area is clean so ensure you are also clean when obtaining necessary equipment.

Figure 6-1 Dissection tools

The necessary dissection tools include:

- Scissors

- Tweezers
- Forceps
- Ruler
- Marker
- Scalpel blade and handle
- Sharp probe

Other necessary tools (not for the dissection):

- Aluminum plate with tapped hole (custom build half inch aluminum anodized plate)
    - Anodized aluminum plate is necessary because the biological materials can't get trapped in the pores of the metal
- Beaker stands with clamps
- Extra paper towel
- Grease threaded rod (12mm fine thread)
- 99% isopropyl alcohol
- Water spray bottle
- Cotton Swabs – short
- Wooden tongue depressor

3. Turn on the fume hood. This is done by pressing the button on the wall (make sure to turn this off after the potting procedure). Then remove the chemicals from the cupboards – the PMMA powder is in the irritants, and the liquid catalyst is in the flammables. Make sure to ask for assistance if this is your first time with PMMA.

4. Next, you will need to prepare a pot from PVC piping.

    i. Cut 4" diameter PVC pipe to a height of 6mm. Make sure the edges are clean so as to prevent leakage of dense (dental) stone during potting—cut with a power tool from Engineering Shops.

ii. Place the cut PVC in the vice that is in the lab and drill holes into the side wall at mid height ~3cm around the pot circumference.

iii. Cover drilled holes with tape in order to prevent the denstone from coming out. Medical tape works best because it allows air exposure to decrease dry time while still preventing the dense stone from leaking out.

5. Specimen Bag

Prepare your plastic bag with specimen label. The first will need to be larger which will hold the specimen until testing. Please see the general SOP for the proper labels and disposal guidelines.

6. Specimen Dissection

I. Get specimen from the thaw bucket or fridge (If the specimens were frozen they will need to be removed from the freezer ~ 24 hrs. prior to Dissection). Remove specimen from packaging and place on the counter top by the sink. Using lots of paper towel is recommended to help keep any fluids contained. Using water, remove any debris from the cattle hoof (as it likely has come straight from the field). Use tweezers if necessary to remove any dirt that is strongly adhered to the cattle hoof. Run Hoof under water for approximately 15 mins. This is done to ensure the hoof is at the maximum moisture content.

II. Using clean gloves, clean the sink basin and taps as well as any remaining dirt in the sink.

III. Before beginning dissections make measurements:

i. Measure 1” from the top of the hoof wall and make a mark (the proximal end of B plus 1”). Continue to do this all the way around the circumference of the hoof.

ii. Fill in the marks with a solid line all the way around the hoof. This is the soft tissue line. Everything under this to the corium will need to be dissected. Mark all the way around based on the anterior corium to create a lower dissection line.

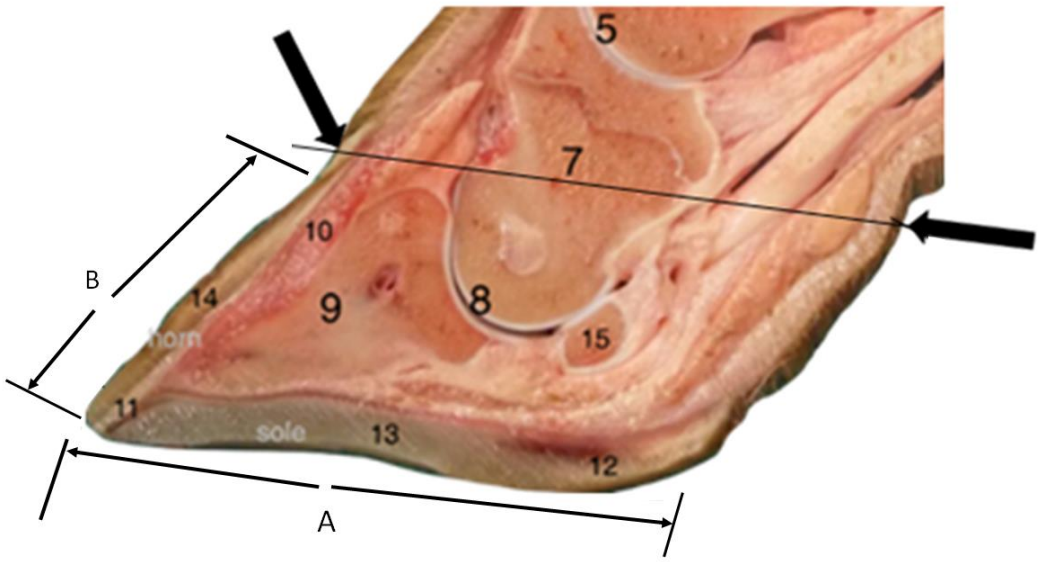

Figure 6-2 Sagittal section through cattle hoof. "A" is the length of the sole. B' is the height of the hoof wall. The line crossing through the P2 (7) bone is the second soft tissue line everything above is dissected.

iii. Using the scalpel, cut along the dissection line. You can use the scissors and the tweezers to help make this cut if it is too difficult.

iv. Remove the skin and soft tissues. The best way to do this is to take the forceps and slowly peel the skin back while using the scalpel to separate skin/fat from the fascia.

** Remember to moisten the specimen to keep the tissues at a maximum moisture content

v. Now the remaining deep tissues can be removed. Carefully cut down to the bone. There should be two bones, each leading up to the main joint at the top of the specimen. Some smaller specimens may have this joint exposed when making this cut. Don't remove the soft tissue from this joint. You can use the sharp probe tool to help locate the joint if you are unsure.

** Be careful not to cut into the phalangeal joint located at the bottom of the P1 bone near the edge of the soft tissue.

Scrape away and soft tissues and periosteum still attached to the bone.

vi. Before potting, take a cotton swab and saturate it with 99% isopropyl alcohol. Use saturated cotton swab to remove any remaining soft tissue that is attached to the bone as well as any remaining oils that will prevent PMMA from adhering

vii. Use PMMA to fix the bones in an anatomical position. This is done by placing the hoof sole down on the counter and using PMMA to fix the hoof in position.

PMMA Ratio: 1 vial of liquid monomer to 1 scoop of PMMA powder.

viii. Let PMMA completely harden.

ix. Use the bone saw to cut off any material above the PMMA. This cut should take place at the Soft tissue line.

7. Potting

Set up the potting station to look like Figure 3. The PVC pot is secured to the aluminum plate using grease, the aluminum plate is placed on the base of the beaker stands, and the clamps are placed on the beaker stand posts (not shown in picture).

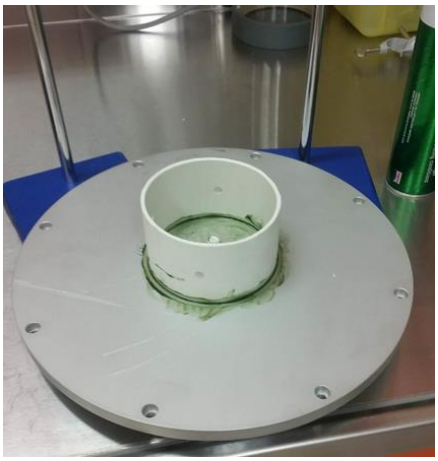

Figure 6-3 PVC pot sealed onto aluminum base with grease.

8. Mark out the location of the pot by marking 2” away from center with a marker. Apply grease to the bottom of the PVC pot and the aluminum plate. Add extra grease to the threaded rod before screwing it into the hole. The threaded rod should come into the pot approximately ½”.
   With clean gloves, use the mixing container and obtain some dry Dental Cement.

9. Using a tongue depressor, mix 3/4 large yogurt container of dental cement powder with enough water to make the consistency of a milkshake. Pour this into the PVC pot so that approximately half the pot is filled. Before the Dental Cement hardens, attach one clamp to each hoof. Ensure the specimen is perpendicular to the work bench. Make sure the specimen corium (B) is in the pot by 0.5cm (the depth can be adjusted by moving the clamps up and down to obtain the correct depth).

   After the specimen is in the pot, make sure there is a small amount of room (~0.5cm) between the top of the pot and the top of the dental cement. If there is a substantial amount of room, then fill the pot with more dental cement. Let dental cement harden.

10. Once dental cement is hard, add PMMA to the top of the dental cement (this should not be much). PMMA must be mixed in the fume hood.

i. PMMA Ratio: 1 scoop of PMMA powder to 1 and 2/3 vile of liquid monomer. Mix for 45-60 sec in a disposable cup.
ii. With clean gloves, get the labeled specimen bag ready and place the specimen into the bag. Remove as much air as possible and heat seal the bag closed. Clean the outside of the bag with bleach spray (sodium dichloro-isocyanurate). The specimen is ready for imaging.
Place all the dissected tissue and bone into the yellow bag in the grey bin.

B. Statistical Analysis

B.1 Objective 1 Statistical Analysis Compressive Loading

Repeated Measures Objective 1, Effects of load on separation area in diseased hooves

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.	Noncent. Parameter	Observed Power[c]
load	Pillai's Trace	.972	26.042[b]	4.000	3.000	.012	104.169	.960
	Wilks' Lambda	.028	26.042[b]	4.000	3.000	.012	104.169	.960
	Hotelling's Trace	34.723	26.042[b]	4.000	3.000	.012	104.169	.960
	Roy's Largest Root	34.723	26.042[b]	4.000	3.000	.012	104.169	.960

a. Design: Intercept
Within Subjects Design: load

b. Exact statistic

c. Computed using alpha = .05

Pairwise Comparisons

Measure: MEASURE_1

(I) load	(J) load	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b] Lower Bound	Upper Bound
1	2	-18.328*	2.006	.001	-26.988	-9.668
	3	-46.096*	5.439	.001	-69.577	-22.616
	4	-66.460*	9.026	.003	-105.424	-27.496
	5	-112.853*	11.698	.001	-163.350	-62.355
2	1	18.328*	2.006	.001	9.668	26.988
	3	-27.768*	4.443	.008	-46.947	-8.589
	4	-48.132*	7.551	.007	-80.730	-15.534
	5	-94.524*	10.671	.001	-140.591	-48.458
3	1	46.096*	5.439	.001	22.616	69.577
	2	27.768*	4.443	.008	8.589	46.947
	4	-20.364	5.192	.078	-42.777	2.049
	5	-66.756*	9.573	.004	-108.081	-25.431
4	1	66.460*	9.026	.003	27.496	105.424
	2	48.132*	7.551	.007	15.534	80.730
	3	20.364	5.192	.078	-2.049	42.777
	5	-46.393*	6.838	.005	-75.910	-16.875
5	1	112.853*	11.698	.001	62.355	163.350
	2	94.524*	10.671	.001	48.458	140.591
	3	66.756*	9.573	.004	25.431	108.081
	4	46.393*	6.838	.005	16.875	75.910

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Repeated Measures Objective 1 healthy hooves

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.	Partial Eta Squared
load	Pillai's Trace	.669	3.032[b]	4.000	6.000	.109	.669
	Wilks' Lambda	.331	3.032[b]	4.000	6.000	.109	.669
	Hotelling's Trace	2.021	3.032[b]	4.000	6.000	.109	.669
	Roy's Largest Root	2.021	3.032[b]	4.000	6.000	.109	.669

a. Design: Intercept
Within Subjects Design: load

b. Exact statistic

Pairwise Comparisons

Measure: MEASURE_1

(I) load	(J) load	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a] Lower Bound	Upper Bound
1	2	-.045	.032	1.000	-.162	.072
	3	-.016	.025	1.000	-.107	.075
	4	-.083	.024	.079	-.173	.007
	5	-.078	.038	.704	-.219	.063
2	1	.045	.032	1.000	-.072	.162
	3	.029	.027	1.000	-.070	.129
	4	-.038	.037	1.000	-.176	.100
	5	-.033	.024	1.000	-.121	.054
3	1	.016	.025	1.000	-.075	.107
	2	-.029	.027	1.000	-.129	.070
	4	-.067	.032	.633	-.184	.050
	5	-.063	.029	.590	-.170	.044
4	1	.083	.024	.079	-.007	.173
	2	.038	.037	1.000	-.100	.176
	3	.067	.032	.633	-.050	.184
	5	.004	.045	1.000	-.160	.169
5	1	.078	.038	.704	-.063	.219
	2	.033	.024	1.000	-.054	.121
	3	.063	.029	.590	-.044	.170
	4	-.004	.045	1.000	-.169	.160

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

Factorial Repeated Measures Analysis

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
load	Pillai's Trace	.953	61.311[b]	4.000	12.000	.000
	Wilks' Lambda	.047	61.311[b]	4.000	12.000	.000
	Hotelling's Trace	20.437	61.311[b]	4.000	12.000	.000
	Roy's Largest Root	20.437	61.311[b]	4.000	12.000	.000
load * group	Pillai's Trace	.953	61.108[b]	4.000	12.000	.000
	Wilks' Lambda	.047	61.108[b]	4.000	12.000	.000
	Hotelling's Trace	20.369	61.108[b]	4.000	12.000	.000
	Roy's Largest Root	20.369	61.108[b]	4.000	12.000	.000

a. Design: Intercept + group
Within Subjects Design: load

b. Exact statistic

3. group * load

Measure: MEASURE_1

group	load	Mean	Std. Error	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
diseased	1	26.823	1.067	24.548	29.098
	2	45.151	1.257	42.472	47.830
	3	72.919	2.953	66.625	79.213
	4	93.283	5.261	82.070	104.495
	5	139.675	7.208	124.312	155.038
healthy	1	.020	.893	-1.883	1.924
	2	.065	1.052	-2.176	2.306
	3	.036	2.471	-5.230	5.302
	4	.103	4.401	-9.278	9.484
	5	.099	6.030	-12.755	12.952

Student's t-tests

T-Test

Group Statistics

	specimen	N	Mean	Std. Deviation	Std. Error Mean
Area1	c	10	.0201890034	.0445508382	.0140882120
	d	11	29.11711357	12.10918415	3.651056395
Area2	c	10	.0652061856	.0857046499	.0271021900
	d	11	52.24038160	16.06777683	4.844616995
Area3	c	10	.0358247423	.0770369979	.0243612377
	d	11	77.83831773	18.47863229	5.571517269
Area4	c	10	.1030927835	.1050079834	.0332064400
	d	10	101.2631091	24.74380202	7.824677236
Area5	c	10	.0986254296	.1107916606	.0350353993
	d	7	139.6753821	30.15176464	11.39629583

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Area1	Equal variances assumed	7.732	.012	-7.580	19	.000	-29.0969246	3.838433610	-37.1308584	-21.0629907
	Equal variances not assumed			-7.969	10.000	.000	-29.0969246	3.651083576	-37.2320129	-20.9618362
Area2	Equal variances assumed	7.338	.014	-10.244	19	.000	-52.1751754	5.093283559	-62.8355404	-41.5148104
	Equal variances not assumed			-10.770	10.001	.000	-52.1751754	4.844692803	-62.9697321	-41.3806187
Area3	Equal variances assumed	10.798	.004	-13.283	19	.000	-77.8024930	5.857465300	-90.0623088	-65.5426772
	Equal variances not assumed			-13.964	10.000	.000	-77.8024930	5.571570528	-90.2166614	-65.3883246
Area4	Equal variances assumed	26.630	.000	-12.928	18	.000	-101.160016	7.824747697	-117.599201	-84.7208314
	Equal variances not assumed			-12.928	9.000	.000	-101.160016	7.824747697	-118.860728	-83.4593044
Area5	Equal variances assumed	12.703	.003	-14.852	15	.000	-139.576757	9.397721455	-159.607526	-119.545988
	Equal variances not assumed			-12.247	6.000	.000	-139.576757	11.39634969	-167.462492	-111.691021

Student's t-tests 3D Imaging

Group Statistics

	specimen	N	Mean	Std. Deviation	Std. Error Mean
volume	c	9	.1423678123	.2481815400	.0827271800
	d	10	304.5605752	256.7454913	81.19005315

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
volume	Equal variances assumed	51.784	.000	-3.547	17	.002	-304.418207	85.83320827	-485.510447	-123.325967
	Equal variances not assumed			-3.749	9.000	.005	-304.418207	81.19009530	-488.082905	-120.753510

B.2 Objective 2 Statistical Analysis Fatigue Loading

Repeated Measures ANOVA assessing the effect of cyclic loading on white line separation of damaged hooves

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
load	Pillai's Trace	.496	1.475[b]	4.000	6.000	.319
	Wilks' Lambda	.504	1.475[b]	4.000	6.000	.319
	Hotelling's Trace	.984	1.475[b]	4.000	6.000	.319
	Roy's Largest Root	.984	1.475[b]	4.000	6.000	.319

a. Design: Intercept
Within Subjects Design: load

Pairwise Comparisons

Measure: MEASURE_1

(I) load	(J) load	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a] Lower Bound	Upper Bound
1	2	.004	.019	1.000	-.067	.074
	3	-.009	.023	1.000	-.094	.076
	4	-.013	.025	1.000	-.107	.080
	5	-.059	.026	.461	-.154	.035
2	1	-.004	.019	1.000	-.074	.067
	3	-.012	.013	1.000	-.062	.037
	4	-.017	.016	1.000	-.075	.041
	5	-.063	.027	.466	-.164	.038
3	1	.009	.023	1.000	-.076	.094
	2	.012	.013	1.000	-.037	.062
	4	-.004	.022	1.000	-.085	.076
	5	-.051	.033	1.000	-.172	.071
4	1	.013	.025	1.000	-.080	.107
	2	.017	.016	1.000	-.041	.075
	3	.004	.022	1.000	-.076	.085
	5	-.046	.027	1.000	-.147	.054
5	1	.059	.026	.461	-.035	.154
	2	.063	.027	.466	-.038	.164
	3	.051	.033	1.000	-.071	.172
	4	.046	.027	1.000	-.054	.147

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

Repeated Measures ANOVA assessing the effect of cyclic loading on white line separation of healthy hooves

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
load	Pillai's Trace	.630	2.551[b]	4.000	6.000	.147
	Wilks' Lambda	.370	2.551[b]	4.000	6.000	.147
	Hotelling's Trace	1.701	2.551[b]	4.000	6.000	.147
	Roy's Largest Root	1.701	2.551[b]	4.000	6.000	.147

a. Design: Intercept
Within Subjects Design: load

b. Exact statistic

Pairwise Comparisons

Measure: MEASURE_1

(I) load	(J) load	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a] Lower Bound	Upper Bound
1	2	-.054	.040	.214	-.145	.037
	3	-.063	.035	.107	-.142	.017
	4	-.035	.038	.379	-.121	.051
	5	-.084	.060	.192	-.219	.051
2	1	.054	.040	.214	-.037	.145
	3	-.009	.043	.843	-.105	.087
	4	.019	.028	.519	-.045	.083
	5	-.030	.064	.651	-.175	.115
3	1	.063	.035	.107	-.017	.142
	2	.009	.043	.843	-.087	.105
	4	.028	.021	.214	-.019	.074
	5	-.021	.057	.716	-.149	.107
4	1	.035	.038	.379	-.051	.121
	2	-.019	.028	.519	-.083	.045
	3	-.028	.021	.214	-.074	.019
	5	-.049	.061	.446	-.188	.090
5	1	.084	.060	.192	-.051	.219
	2	.030	.064	.651	-.115	.175
	3	.021	.057	.716	-.107	.149
	4	.049	.061	.446	-.090	.188

Based on estimated marginal means

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Factorial Repeated Measures ANOVA Damaged vs. Healthy white line separation

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
time	Pillai's Trace	.282	1.473[b]	4.000	15.000	.259
	Wilks' Lambda	.718	1.473[b]	4.000	15.000	.259
	Hotelling's Trace	.393	1.473[b]	4.000	15.000	.259
	Roy's Largest Root	.393	1.473[b]	4.000	15.000	.259
time * Group	Pillai's Trace	.233	1.139[b]	4.000	15.000	.376
	Wilks' Lambda	.767	1.139[b]	4.000	15.000	.376
	Hotelling's Trace	.304	1.139[b]	4.000	15.000	.376
	Roy's Largest Root	.304	1.139[b]	4.000	15.000	.376

a. Design: Intercept + Group
Within Subjects Design: time

b. Exact statistic

Tests of Within-Subjects Effects

Measure: MEASURE_1

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
time	Sphericity Assumed	.054	4	.014	1.969	.108
	Greenhouse-Geisser	.054	2.514	.022	1.969	.141
	Huynh-Feldt	.054	3.117	.017	1.969	.127
	Lower-bound	.054	1.000	.054	1.969	.178
time * Group	Sphericity Assumed	.012	4	.003	.418	.795
	Greenhouse-Geisser	.012	2.514	.005	.418	.706
	Huynh-Feldt	.012	3.117	.004	.418	.748
	Lower-bound	.012	1.000	.012	.418	.526
Error(time)	Sphericity Assumed	.497	72	.007		
	Greenhouse-Geisser	.497	45.247	.011		
	Huynh-Feldt	.497	56.110	.009		
	Lower-bound	.497	18.000	.028		

2. Group * time

Measure: MEASURE_1

Group	time	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
damage	1	.014	.022	-.033	.062
	2	.011	.028	-.048	.070
	3	.023	.038	-.056	.102
	4	.027	.035	-.045	.100
	5	.074	.044	-.019	.167
healthy	1	.063	.022	.016	.110
	2	.117	.028	.058	.176
	3	.126	.038	.047	.205
	4	.098	.035	.026	.171
	5	.147	.044	.054	.240

Paired t-tests Fatigue Loading

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	Area1c - Area1	.0485395189	.0889139519	.0281170604	-.015065691	.1121447284	1.726	9	.118
Pair 2	Area2c - Area2	.1060996564	.1290905832	.0408220267	.0137538162	.1984454965	2.599	9	.029
Pair 3	Area3c - Area3	.1024914089	.1826333097	.0577537235	-.028156590	.2331394083	1.775	9	.110
Pair 4	Area4c - Area4	.0706185567	.1147262352	.0362796211	-.011451648	.1526887613	1.947	9	.083
Pair 5	Area5c - Area5	.0731958763	.2055744571	.0650083513	-.073863231	.2202549839	1.126	9	.289

Paired t-tests 3D Imaging

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	damaged - healthy control	-.013753568	.3231866452	.1077288817	-.262176815	.2346696783	-.128	8	.902